INTRINSIC FORMS OF ACETYLCHOLINESTERASE IN SKELETAL MUSCLE

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1. Introduction

Acetylcholinesterase (AChE; EC 3.1.1.7) occurs in mammalian and avian skeletal muscle in a number of molecular forms as characterised by their sedimentation coefficients on sucrose gradients. In rat diaphragm, three principal forms were observed by Hall [1], who showed that the heaviest (H) form, 16 S AChE, was specifically associated with the endplate regions of the muscle and was greatly reduced in amount upon denervation. Vigny et al. [2,3] provided additional evidence suggesting that the H form was endplate-specific in various muscles of the rat and the chicken. In all 3 studies, however, the H form accounted for only a minor part of the total AChE activity, the greater part of the enzyme occurring in peaks of medium (M) or low (L) sedimentation coefficient. Treatment with collagenase or proteases can modify the molecular forms of AChE in skeletal muscle [4–6], and it has been suggested that endogenous proteases may affect the distribution of AChE species observed in skeletal muscle extracts [5,7]. In the present study, therefore, we have examined the effect of certain protease inhibitors on the sedimentation profile of AChE activity in extracts of various chicken muscles and of rat diaphragm. We will demonstrate that use of protease inhibitors in the extraction medium prevents degradation of the intrinsic molecular forms of AChE and greatly simplifies the sedimentation profiles obtained. Using an appropriate mixture of inhibitors, it is shown that AChE in normal pectoral muscle is predominantly of the H (19.5 S) type. In the pectoral muscle of dystrophic chickens the major forms are the H form and an L (6.5 S) species.

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2. Materials and methods

2.1. *Chemicals*

Acetylthiocholine iodide, 5,5′-dithiobis(2-nitrobenzoic acid) (DNTB), beef liver catalase, EGTA, *N*-ethylmaleimide, benzamidine hydrochloride and bacitracin were obtained from Sigma Chemicals; tetraisopropylpyrophosphoramide (iso-OMPA) from Koch-Light and Triton X-100 from Rohm and Haas. Pepstatin and leupeptin were provided by the USA–Japan Cooperative Cancer Research Program.

2.2. *Preparation of muscle tissue extracts*

Tissue extracts were made from muscles of freshly sacrificed animals (normal (line 412) or dystrophic (line 413) chickens from the University of California (Davis) flock, or Sprague-Dawley rats). Samples (~100 mg) of the muscle were homogenised in 1 ml ice-cold extraction buffer in a glass–glass hand homogenizer. The homogenate was centrifuged at 15 000 rev./min for 30 min in an SS-34 rotor in a Sorvall RC-5 centrifuge at 4°C, and the pellet discarded. The extraction media used were either the control medium, which was composed of 1% Triton X-100/1 M NaCl/0.01 M phosphate, pH 7.0, or the antiprotease medium which contained, in addition, 10 mM EGTA, 2 mM benzamidine hydrochloride, 5 mM *N*-ethylmaleimide, 40 μg/ml leupeptin and 20 μg/ml pepstatin, and was prepared immediately before use. Where stated, bacitracin (1 mg/ml) was also included in the antiprotease medium.

2.3. *Acetylcholinesterase assays*

AChE assays were performed by the method in [8] in a reaction medium containing 0.1 mM iso-OMPA/1 mM DTNB/0.1% Triton X-100/0.1 M phosphate, pH 7.0. After preincubation at 25°C for 30 min to permit full inactivation of pseudocholinesterase (acylcholine hydrolase; EC 3.1.1.8) by the iso-OMPA [9], the reaction was initiated by addition of acetylthiocholine iodide to final 0.75 mM.

2.4. *Sucrose gradient centrifugation*

Centrifugation was performed on 5–20% sucrose gradients layered over a 60% sucrose cushion (0.5 ml) using 12 ml tubes in a SW40Ti rotor, at 40 000 rev./min for 17 h in a Beckman L5-65 ultracentrifuge at 4°C. The sucrose solutions were made up in 1 M NaCl/0.1% Triton X-100/0.01 M phosphate, pH 7.0. Beef liver catalase (11.4 S) was the principal marker.

3. Results

3.1. *Chicken muscles*

Sucrose gradient centrifugation of chicken skeletal muscle reveals AChE activity to be principally concentrated in 3 zones of the gradient, arbitrarily designated as heavy (H, 15–20 S), medium (M, 10–13 S) and light (L, 4–7 S). Figure 1a shows a typical gradient obtained with pectoral muscle from a day 29 dystrophic chicken extracted in 1% Triton/1 M NaCl in the absence of protease inhibitors. It is known that in fast muscles of dystrophic chickens AChE levels are greatly elevated [10], and it has recently been shown that this elevation is largely accounted for by an increase in the L forms of the enzyme [11]. Indeed it can be seen that there are considerable amounts of two light forms, L_1 (~4.5 S) and L_2 (~6.5 S), as well as of one heavy (H_2, ~19 S) form. However, if extraction is similarly performed at identical salt and detergent concentrations but in the presence of protease inhibitors (fig.1b) the H_2 and L_2 components become much more pronounced and the L_1 component is almost totally absent. The effect of the protease inhibitors is further emphasised if the corresponding extracts are incubated for 1 h at 38°C before application to the gradient. In the absence of protease inhibitors (fig.1c) the H_2 and L_2 forms are almost completely eliminated, over 40% activity

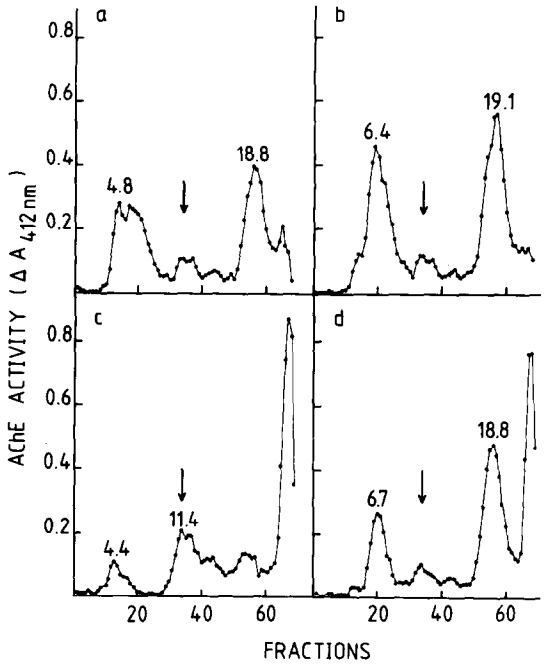

Fig.1. Sucrose gradient profiles of AChE activity in extracts of pectoral muscle of a day 29 dystrophic chicken. (a) Extraction in control medium. (b) Extraction in antiprotease medium. (c) Extraction in control medium followed by incubation for 1 h at 38°C. (d) Extraction in antiprotease medium followed by incubation for 1 h at 38°C. The arrows denote the position of the catalase marker.

appears as large aggregates, the M forms are elevated and small amounts of L_1 are observed. At 38°C, in the presence of protease inhibitors (fig.1d), although 30% activity is still in the aggregates, the H_2 peak is only slightly diminished, there is no increase in the M region and, while the L_2 form is greatly reduced compared to an untreated sample, no concomitant appearance of L_1 occurs. It should be emphasised that the incubation at 38°C produces no significant decrease in the overall AChE activity of either the control or antiprotease extracts.

Figure 2a demonstrates that in young (day 7) normal chickens several molecular forms of AChE are also observed even in the presence of protease inhibitors, the major components being H_2 and M, with H_1 (15.7 S) and L_2 also present; L_1 is almost entirely absent. If, however, an extract from a more mature (e.g., day 32) normal bird, made in the presence of

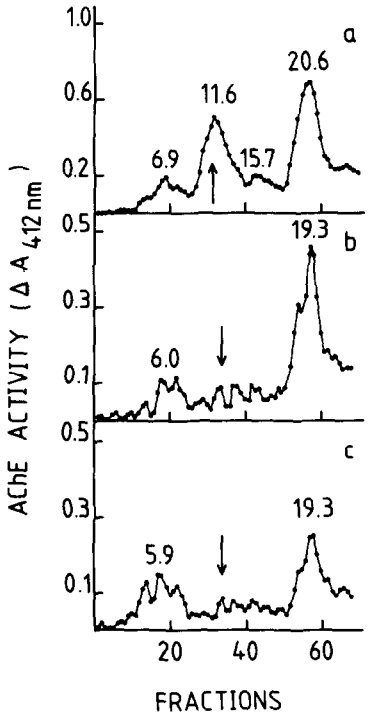

Fig.2. Sucrose gradient profiles of AChE activity in extracts of pectoral muscle of normal chickens. (a) Extract of day 7 chicken in antiprotease medium including bacitracin. (b) Extract of day 32 chicken in antiprotease medium. (c) Extract of day 32 chicken in control medium. The arrows denote the position of the catalase marker.

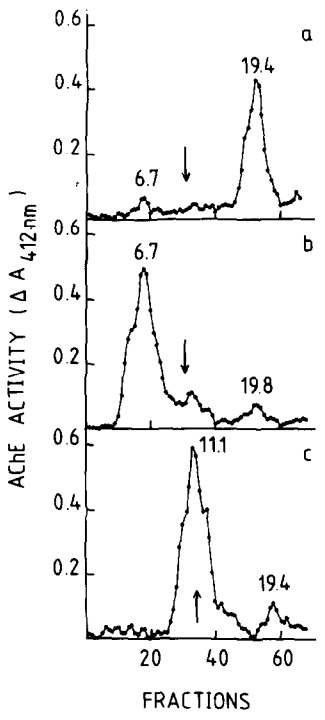

Fig.3. Sucrose gradient profiles of AChE activity in extracts of PLD, ALD and cardiac muscles from normal chickens. (a) PLD from day 60 chicken in antiprotease medium containing bacitracin. (b) ALD from day 60 chicken in antiprotease medium containing bacitracin. (c) Cardiac muscle from day 32 chicken in antiprotease medium. The arrows denote the position of the catalase marker.

protease inhibitors, is similarly analysed (fig.2b), the H_2 form is found to be predominant, L forms being present to only a minor extent and M forms almost completely absent. If such a muscle is similarly extracted without protease inhibitors, the H_2 peak is greatly decreased in magnitude, and lighter forms account for a large part of the activity seen on the gradients (fig.2c). Prolonged storage of such homogenates at −20°C, or of frozen tissue prior to homogenisation, further reduce the level of H_2 while lighter forms become more prominent (not shown).

Figure 3a shows that in the presence of protease inhibitors during extraction, another singly-innervated fast muscle, the posterior latissimus dorsi (PLD), also displays only one major AChE component, the H_2 form, in normal adult chickens. This does not, however, hold true for all skeletal muscles. The multiply-innervated slow muscle, the anterior latissimus dorsi (ALD), contains only minor amounts of the H and M forms, even when freshly homogenised in the presence of the protease inhibitors, the major species being the L_2 (6.5 S) form (fig.3b). Cardiac muscle presents yet again a different pattern, almost all AChE activity being concentrated in the M region of the gradient (fig.3c).

3.2. *Rat muscles*

The effects of protease inhibitors on the distribution of molecular forms of AChE in skeletal muscle is not confined to the chicken. If sucrose gradient centrifugation is performed on extracts of the endplate regions of rat diaphragm made in the presence and absence of protease inhibitors, two very different patterns are obtained. In the absence of the inhibitors

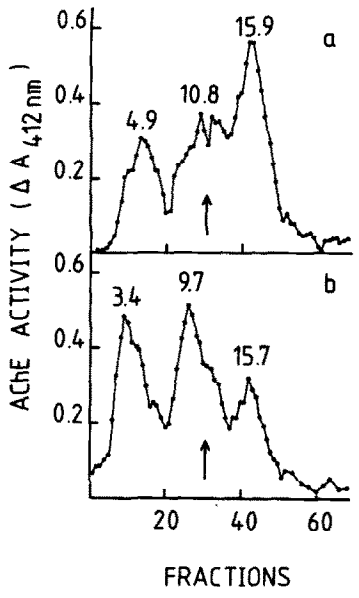

Fig.4. Sucrose gradient profiles of AChE activity in extracts of endplate regions dissected from the diaphragms of month 6 rats. (a) Extraction in antiprotease medium containing bacitracin. (b) Extraction in control medium. The arrows denote the position of the catalase marker.

the H (16 S) form is a minor component compared to the L and M species (fig.4b), whereas in their presence it is the major form observed (fig.4a). Inspection of fig.4a,b also reveals that in the presence of the protease inhibitors heavier components in both the M and L zones of the gradient predominate, whereas lighter forms are more prevalent in both zones in the absence of inhibitors.

4. Discussion

The results presented illustrate two principal points:

1. The use of suitable protease inhibitors in the preparation of skeletal muscle extracts markedly retards the modification of intrinsic forms of AChE present in the tissue.
2. Although multiple molecular forms of AChE are indeed found in muscle tissue, when a given muscle type from an adult is examined under conditions where degradation is largely prevented, the distribution of molecular forms is, in fact, much simpler than was thought previously, a single molecular form frequently predominating.

In the experiments described, various protease inhibitors were used. Some of these inhibitors, e.g., benzamidine, EGTA and *N*-ethylmaleimide, have been used in the isolation of procollagen, where rigorous inhibition of proteolysis is needed to prevent its conversion to collagen (see e.g., [12]). In addition, EGTA should serve to inhibit the Ca^{2+}-activated muscle protease [13], while *N*-ethylmaleimide also markedly improves the resolution of the gradients by alkylating free sulfhydryls on extraneous proteins in the muscle extracts, which otherwise produce background colour by reduction of DTNB. Bacitracin, which was used in some experiments, has been recommended [14] because of its potent effect in preventing degradation of glucagon by microsomal extracts. The two other polypeptide inhibitors, leupeptin and pepstatin, in combination, have been shown to be very effective in blocking the action of lysosomal proteases from rat liver, the additional presence of an alkylating agent suppressing proteolysis completely [15,16]. It has recently been shown that these latter reagents inhibit similarly the lysosomal proteases of rat skeletal muscle [17]. It thus seems most probable that extraction in the antiprotease medium employed preserves the intrinsic pattern of molecular forms of AChE present in intact skeletal muscle. The latter conclusion is supported by the results of the autolysis experiments shown in fig.1c,d. At 38°C, both in control and antiprotease extraction media, considerable aggregation of AChE occurred. Nevertheless, in the antiprotease medium, little degradation of the H_2 form was observed, nor were the L_1 form or M forms produced in significant amounts. The aggregation phenomenon must therefore result either from direct physical aggregation of the L_2 species or from some other form of enzymic action (e.g., of glycosidases or phospholipases).

Using our protease inhibitor extraction medium, it immediately becomes apparent that the only major AChE species present in two different fast-twitch muscles, pectoral and PLD of the normal chicken, is the H_2 form. In adult dystrophic chickens large amounts of the L_2 (6.5 S) form are also observed, which are unlikely to be formed from the H form by degradation in vitro, but which might be so formed in

the dystrophic condition in vivo. Alternatively, this elevation of the L_2 form may result from direct overproduction. In the slow-twitch muscle (ALD), H and M forms are present to only a minor extent, L_1 and L_2 forms predominating. Since the H form may comprise as little as 10% total activity, the possibility must be considered that it is not the functional species at the multiple endplates. It is also detected in small amounts in extracts of cardiac muscle, which is devoid of conventional endplates. Since it has been demonstrated that H forms of AChE are transported down the sciatic nerve by rapid axonal flow [18], it is possible that the H_2 AChE detected in ALD and cardiac muscle derives from the adhering innervation.

In the presence of protease inhibitors, a large part of the total AChE activity in the rat diaphragm consists of L and M forms. However, in the endplate region the H form accounts for as much as 40–50% total, and this figure might be higher were it possible to examine endplates in complete isolation.

From the above results and discussion it is apparent that considerable care must be taken in analysing the distribution of AChE forms in muscle (or indeed in any tissue) and in assigning functional significance to all of the species observed.

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